International Journal of Biochemistry & Cell Biology (1999) *31* 8: 869-882.doi: 10.1016/S1357-2725(99)00038-2

Molecular cloning, expression, localisation and functional characterisation of a rabbit SULT1C2 sulfotransferase

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Abstract

The importance of sulfotransferases in xenobiotic metabolism is gaining recognition. The gastrointestinal (GI) tract is a major portal of entry for many xenobiotics, yet little is known about the contribution of sulfotransferases to detoxication or bioactivation metabolism in these tissues. To this end, isolation and characterisation of sulfotransferases expressed in the stomach of rabbits was undertaken. A unique sulfotransferase cDNA (GenBank Accession No. AF026304) was isolated from a rabbit stomach cDNA library. This cDNA was 1439 base pairs (bp) long and has an open reading frame of 888 bp. On expression of the cDNA in both COS cells and E. coli, a protein molecular weight of 34 kDa was detected on SDS-PAGE. Immunoblotting using an antibody raised in goats against the bacterially expressed protein detected expression of the protein in GI tract tissues. The 34 kDa immunoreactive band was detected in rabbit GI tract tissues (stomach, duodenum, jejunum, ileum, colon, caecum and rectum), liver and kidneys, but not in the lungs (n= 3). The human ortholog (GenBank Accession No AF026303) of the rabbit enzyme was cloned from a human stomach cDNA library. These two enzymes share 84% amino acid sequence identity and have been termed 1C2 sulfotransferases. When functional and kinetic characterisation of the recombinant rabbit and human proteins was carried out using 16 known ST substrates, detectable sulfonation activity was observed only with p-nitrophenol (with Km values of 2.2 mM and 13.3 mM, respectively). In conclusion, we have identi®ed a rabbit GI tract sulfotransferase belonging to a newly defined sulfotransferase subfamily.

Keywords: Gastrointestinal tract; Human; Stomach; Sulfotransferase

Abbreviations: GI, gastrointestinal; PAPS, 3'-phosphoade- nosine-5'-phosphosulfate; PST, phenol sulfotransferase; ST, sulfotransferase.

Introduction

Conjugation with sulfonate is an important pathway involved in the biotransformation of numerous drugs and xenobiotics. In most cases sulfonation is a detoxification pathway but in the case of the antihypertensive drug minoxidil, *N*-hydroxy heterocyclic and aryl amines, and polycyclic aromatic hydroxymethylarenes, it leads to biological activation. Activation of *N*-hydroxy heterocyclic and aryl amines and polycyclic aromatic hydroxymethylarenes leads to highly reactive electrophiles that are both mutagenic and carcinogenic [1]. The sulfonation reaction is catalysed by an emerging supergene family of enzymes termed sulfotransferases, with 3′-phosphoadenosine-5′-phosphosulfate (PAPS) acting as the sulfuryl donor [2]. Prior to 1997, more than 30 different forms of cytosolic sulfotransferases (ST) had been characterised from organisms ranging from microbes to humans [3]. Interrogation of ST sequences submitted to GenBank now show that this number has now risen to well over 50. These enzymes have been characterised into four gene families: SULT1, SULT2 and SULT3 in mammals and

SULT200 in plants. The SULT1 family has four subfamilies that predominantly exhibit activity towards phenolic compounds. The SULT2 family is comprised of two subfamilies that catalyse mainly the metabolism of hydroxysteroids. The first member of the SULT3 gene family was recently characterised by Yoshinari et al. [4] and catalyses the sulfonation of amines. The plant SULT200 sulfotransferase family exhibits a preference for flavonoid substrates.

Members of the SULT1 family have been shown to play a major role in the metabolism of most xenobiotic and endobiotic compounds including simple planar phenols such as napthol and *p*-nitrophenol, drugs such as acetaminophen and endogenous compounds such as catecholamines and thyroid hormones. As indicated above four distinct subfamilies of the SULT1 family have been identified, SULT1A (phenol ST), SULT1B (dopa/tyrosine ST), SULT1C (*N*-hydroxyarylamine ST) and SULT1E (estrogen ST) [5]. Despite high sequence identity, these isozymes differ markedly in their substrate specificity, inhibitor sensitivity and regulation [5 and 6]. Their importance in the metabolism of drugs and xenobiotics is reflected by their extensive distribution and abundance in mammalian tissues and their broad substrate specificities.

In this paper we report the isolation and characterisation of a new rabbit member of the SULT1C subfamily from a stomach λZap cDNA library. In the rabbit to date two cytosolic sulfotransferases have been characterised. These are a rabbit member of the hydroxysteroid sulfotransferase family (SULT2A8) and a form which catalyses the metabolism of amines (SULT3A1). During the course of these studies we also isolated the human ortholog of the rabbit SULT1C cDNA from a human stomach cDNA library. Both of these enzymes can be arbitrarily classified as SULT1C2 members of the SULT1C subfamily. In this paper we also report the tissue distribution and catalytic characterisation of the rabbit SULT1C2 enzyme. The ability of the humSULT1C2 to metabolise a range of substrates was also investigated. The recently introduced ST nomenclature is used in this report [3][1].

Materials and Methods

Materials

The pCMV5 COS expression vector was kindly provided by Dr Mark Stinsky, Department of Microbiology, School of Medicine, University of Iowa (IA, USA). Restriction enzymes were purchased from New England Biolabs (MA, USA). 4-Acetaminophenol, allopurinol, 7-OH-2-acetylaminofluorine (AAF), bromophenol blue, 4-chloro-1-napthol, chlorozoxazone, dexamethasone, dopamine, indole-3-carbinol, minoxidil, 3′-phosphoadenosine-5′-phosphosulfate (PAPS), pargyline, p-nitrophenol, poly-EAY (Glu, Ala, Tyr, 6:3:1) and thyroxine were purchased from Sigma Chemical Company (MO, USA). ^{3}H-estrone and ^{3}H-dehydroepiandrosterone (DHEA) were purchased from Du Pont (DE, USA) and 4-hydroxytamoxifen was from Calbiochem (NSW, Australia). [^{35}S]PAPS was purchased from Amrad Pharmacia Biotechnology (Vic., Australia), 2,6-dichloro-4-nitrophenol (DCNP) from ICN Biochemicals (NSW, Australia) and nitrocellulose membrane from Schleicher and Schuell (Dassel, Germany). All other reagents were of molecular biology or analytical reagent grade.

Isolation of the full length cDNA from rabbit stomach

A rabbit stomach cDNA library was constructed as described previously [7] in bacteriophage λZAPII containing pBluescript vector (Stratagene, La Jolla, CA, USA) and was a kind gift from Dr George Sachs, Wadsworth Veterans Administrative Hospital, University of California (LA, USA). PCR analysis of serial dilutions of the rabbit stomach cDNA library was used to isolate individual clones containing the rabbit cDNA of interest. The PCR primers were designed following alignment of rat phenol [8], rat hydroxysteroid [9] and bovine estrogen [10] sulfotransferase cDNA sequences; forward primer 5′-GTG AAT TCT CTC TTC TAT GAA GAC AT-3′, reverse primer 5′-CTA TCG ATA AGT GAT TCT TCC AGT C-3′. The PCR reactions were performed with 100 μg of DNA template, 100 ng of each primer, 200 μM of each of the four deoxynucleotide triphosphates, 2.5 mM $MgCl_2$, 2 units of Red Hot DNA polymerase and Red Hot DNA polymerase buffer (Integrated Sciences, Sydney, Australia) in a reaction volume of 100 μl. Thirty cycles of the PCR reaction were carried out with denaturation at 94°C for 1 min, annealing at 42°C for 30 s and polymerisation at 72°C for 1 min. Sequential rounds of serial dilution of the cDNA libraries and PCR analysis were undertaken until an individual plaque containing the cDNA of interest was isolated. Positive

[1] In our laboratory the initial names given to the cDNA sequences referred to in this paper are: RbAST5 (rabSULT1C2), HAST1 (humSULT1A1), HAST3 (humSULT1A3) and HAST5 (humSULT1C2).

plasmids were rescued from the phages and ssDNA isolated using the R408 F1 helper phage (Promega, Sydney, Australia) [11]. The rabbit cDNA was isolated within the pBluescript vector associated with the library. The DNA was sequenced in both directions via the dideoxynucleotide chain termination sequencing method of Sanger and Coulson [12] under the conditions recommended in the T7 Sequenase DNA sequencing kit (Amersham, Sydney, Australia).

In parallel to this work, a human stomach Marathon-Ready™ cDNA library purchased from Clontech Laboratories (CA, USA) was also screened using PCR. The same forward primer used in the rabbit work was employed and the reverse primer was 5′-ATC AAA TCG ATC ATT CTG GGC TAC-3′. DNA was prepared from the positive clones identified, digested with the restriction enzymes *Eco*RI and *Xba*I, the cDNA was subcloned into similarly digested M13mp18 vector for sequencing using the method above.

COS-cell expression

The rabbit stomach sulfotransferase cDNA was excised from the pBluescript construct using EcoRI and subcloned into a pCMV5 expression vector digested with the same restriction enzyme. The human stomach ST cDNA was excised from the M13mp18 using EcoRI and XbaI and subcloned into similarly digested pCMV5 expression vector. Competent E. coli HB101 cells (Promega) were transformed with the expression construct and DNA was purified using Wizard JETstar purification columns (Promega). Transfection of COS-7 cells was carried out as described by Veronese et al. [13]. Briefly, COS-7 cells were grown to 80% confluency in Dulbeccos Modified Eagle Media (DMEM) (pH 7.0) containing 10% newborn-calf serum and 50 mg/ml gentamycin. COS-7 cells were transfected with 20 μg DNA using the DEAE–Dextran method, followed by chloroquine treatment (100 μM) for 4 h. Cells were harvested 48 h after transfection, resuspended in 0.1M potassium phosphate buffer (pH 7.0) containing 20 vol% glycerol and stored at −80°C until used. COS-7 cells transfected with pCMV5 lacking the ST inserts were identically prepared as negative controls while previously cloned humSULT1A1 and humSULT1A3 were transfected as positive controls [13].

Polyclonal antibody production

A polyclonal antibody was raised in goats to the recombinant rabbit stomach sulfotransferase (rabSULT1C2). The cDNA was obtained from the pCMV5 construct using PCR with the following primers: forward 5′-CCC TGA GCC CAT ATG GCG CTG GCC ACA G-3′ and reverse 5′-GAT GTG CTT CTC GAG ACT TCT GGC CAC CCT-3′. The PCR product was digested with NdeI and XhoI and subcloned into similarly digested bacterial expression vector pET-20b(+). E. coli bacterial strain BL21 (De3) cells (Novagen, Madison, WI, USA) were transformed with the pET-20b (+)/rabSULT1C2 construct. An overnight culture of transformed cells was used to inoculate 100 ml of SOB medium (per litre, bacto-tryptone 20 g, bacto-yeast extract 5 g, NaCl 0.5 g, KCl 2.5 mM and $MgCl_2$ 2.5 mM) and grown with shaking at 37°C for 16 h. Cultures were harvested and fractionated as described by Sandhu et al. [14]. The supernatant containing the recombinant protein was submitted to SDS-PAGE in 12% acrylamide. The recombinant protein (34 kDa MW band) was excised and used as an antigen to produce anti-(recombinant rabSULT1C2) antisera in goats essentially as described previously [15].

Immunoblot analysis

Proteins were separated using SDS-PAGE in 10% acrylamide according to the method of Laemmli [16], then electrophoretically transferred onto nitrocellulose membranes as previously described [17]. Non-specific protein binding was blocked using buffer A (20 mM Tris–HCl, pH 7.5 containing 20 mM NaCl and 0.05 vol% Tween 20) containing 1% (w/v) skim milk powder. After washing, the membrane was incubated with goat RabSULT1C2 antisera (1:200 dilution in buffer A containing 1 mM $MgCl_2$). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-sheep/goat IgG (1:1500 dilution, Silenus, Vict., Australia) and immuno-reactive protein was detected with 3,3′-diaminobenzidine tetrahydrochloride.

Rabbit tissue cytosol preparation

White New Zealand rabbits were sacrificed by cervical dislocation (University of Queensland AEEC approval number PHYS/PH/421/96/NHMRC/PHD) and tissues quickly removed and frozen in liquid nitrogen before storage at −80°C. All subsequent procedures were undertaken at 4°C. Gastrointestinal tract tissues were thoroughly cleaned

by washing with phosphate-buffered saline (PBS) and scraping the mucosa with a blunt spatula. Mucosal tissue was homogenised (20% w/v in PBS) for 20 s using an Ultra-Turrax homogeniser (Janke & Kunkel Labortechnik, Stanfen, Germany). The homogenate was centrifuged in a Beckman J2-MC centrifuge at 17,000g for 20 min to remove cell debris, nuclei and mitochondria. The supernatant was further centrifuged in a Beckman L-70 ultracentrifuge at 105,000g for 60 min to separate the microsomal fraction. The resulting cytosolic supernatant fraction was stored at −80°C. Cytosolic protein concentration was determined according to the method of Lowry et al. [18] with bovine serum albumin as the standard.

Enzyme assays and kinetic analysis

Enzyme activity was measured by the method of Foldes and Meek [19] with modifications as described by Campbell et al. [20]. The reaction mixture (500 μl) contained 20 mM potassium phosphate buffer (pH 7.0), 8 μM PAPS (of which 0.25 μM was [^{35}S]PAPS) and the substrate. The reaction was initiated by the addition of 30 μg of COS-cell protein (rabSULT1C2) and allowed to proceed for 30 min at 37°C. Assays with dopamine as substrate also included 1 mM of pargyline, a monoamine oxidase inhibitor. Assay blanks were identical except that they lacked the substrate. Control assays using COS cells transfected with vector only were undertaken to confirm a lack of endogenous activity. A range of potential substrates that have previously been shown to be sulfonated by humSULT1A1 [21], humSULT1A3 [22], humSULT2A1 [23] and humSULT1E [24] were tested. In total, 16 different compounds were screened using a range of concentrations (both in micromolar and millimolar ranges) as potential substrates; 4-acetaminophenol, allopurinol, 7-OH-2-acetylaminofluorine (AAF), bromophenol blue, 4-chloro-1-napthol, chlorozoxazone, dexamethasone, DHEA, dopamine, estrone, 4-hydroxytamoxifen, indole-3-carbinol, minoxidil, p-nitrophenol, poly-EAY (Glu, Ala, Tyr, 6:3:1) and thyroxine. Apparent K_m and V_{max} were determined according to the Michaelis–Menten equation using the Graphpad Prism program (Graphpad software, CA, USA).

Thermostability and inhibition studies

Thermostability of the rabbit ST enzyme (COS-expressed and stomach cytosol) was investigated after the enzyme source was pre-incubated for 15 min at temperatures ranging from 35 to 55°C prior to addition to the assay. Sensitivity of the sulfotransferase enzyme to the inhibitor 2,6-dichloro-4-nitrophenol (DCNP) was tested at concentrations ranging from 0.01 to 1000 μM. Both thermostability and inhibitor assays were performed as described above with 2 mM p-nitrophenol as the substrate. Included as positive controls were the COS expressed humSULT1A1 and humSULT1A3 with 2 μM p-nitrophenol and 60 μM dopamine used as substrates, respectively.

Results

Isolation of positive clones

Screening of the rabbit stomach cDNA library using PCR analysis enabled the isolation of a positive clone from the library. DNA sequence analysis of this clone revealed a cDNA of 1439 base pairs in length. The cDNA contained an open reading frame of 888 bp and encoded a protein of 296 amino acids in length. The rabbit cDNA was named rabSULT1C2 to conform to the current ST nomenclature [3]. The PCR screening of the human stomach cDNA library also enabled the isolation of a positive clone from that library. The cDNA sequence was 1027 bp long and contained an open reading frame of 888 bp encoding a protein of 296 amino acids in length.

Sequence analysis

The nucleotide and translated amino acid sequence of rabSULT1C2 is shown in Fig. 1. The first ATG codon required for translation initiation in the open reading frame was located from bases 47 to 50. The third upstream purine from the initiation codons is guanine in the rabbit cDNA. The translation stop codon (TGA) is located from base 935 to 937 and polyadenylation signals (AATAAA) are observed from bases 1385 to 1390. The human sequence reported in this paper is identical to two previous sequences reported by Her et al. [25] and Yoshinari et al. [26].

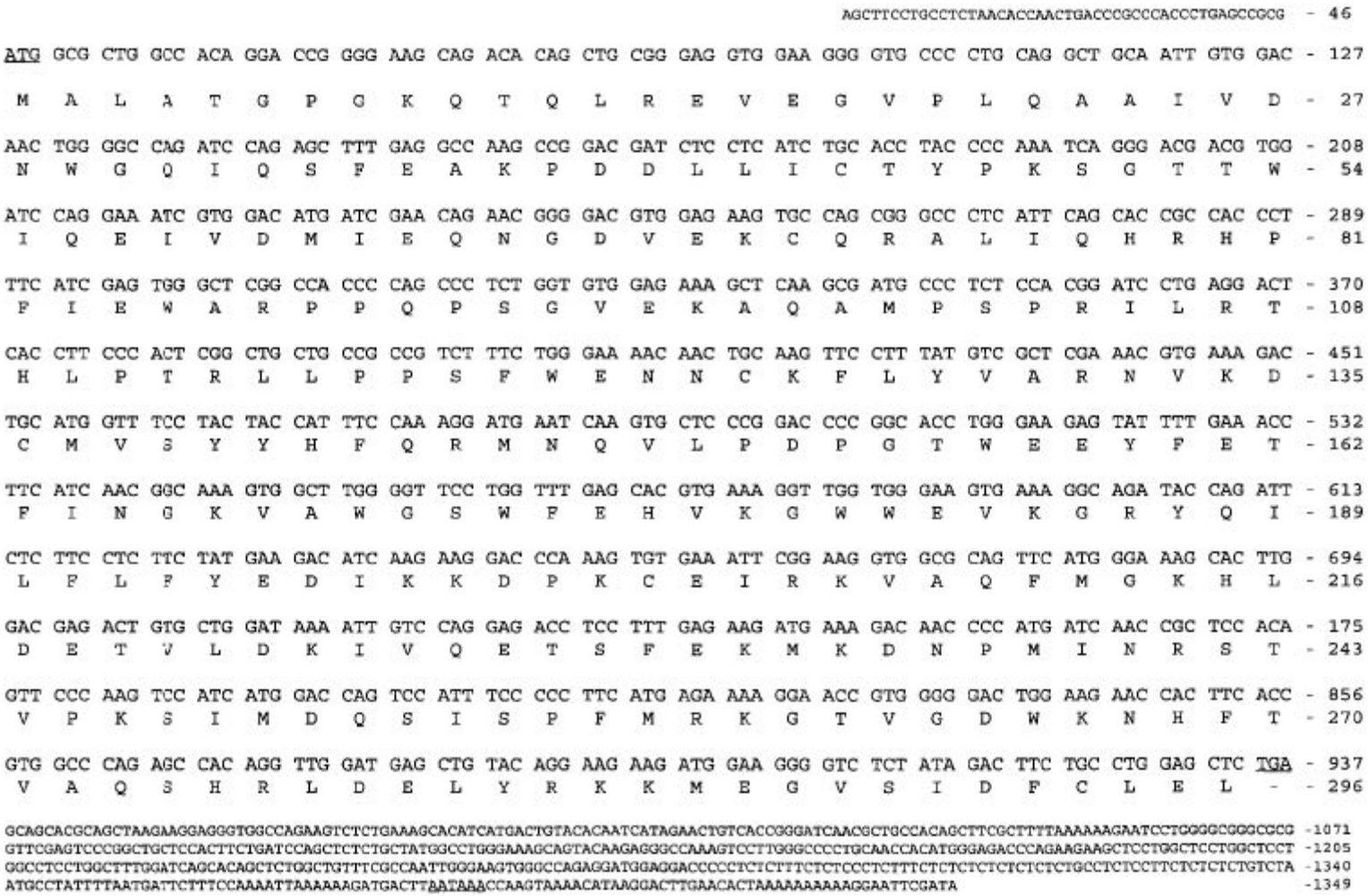

Fig. 1. The nucleotide and deduced amino acid sequences of the rabSULT1C2 cDNA. The ATG start codon, third upstream purine, TGA stop codon and polyadenylation signal AATAAA are underlined. The accession number of this cDNA in the GenBank is AF026304.

Amino acid sequence alignment of rabSULT1C2 and its human ortholog humSULT1C2 is shown in Fig. 2. These enzymes are identified as belonging to the sulfotransferase supergene family by the presence of so-called 'signature sequences' within their primary structure and these regions are shown underlined on Fig. 2. The consensus sequence TYPKSGTxW (where x is any amino acid) is located in both the rabbit and human sequences in the N-terminal region from amino acids 46–54 in a part of the protein structure termed region I [3]. The consensus sequence RKGxxGDWKxxFT is located in a C terminal region (termed region IV [3 and 5]) from amino acids 258–270 for both enzymes. The lack of a hydrophobic transmembrane sequence suggests that rabSULT1C2 and humSULT1C2 are soluble enzymes. The calculated molecular weights of the encoded proteins are 34,572 Da for the rabbit enzyme and 34,910 Da for the human sulfotransferase.

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rabSULT1C2    - MALATGPGKQTQLREVEGVPLQAAIVDNWGQIQSFEAKPDDLLICTYPKS -50
humSULT1C2    - MALTSDLGKQIELKEVEGTLLQPATVDNWSQIQSFEAKPDDLLICTYPKA -50

rabSULT1C2    - GTTWIQEIVDMIEQNGDVEKCQRALIQHRHPFIEWARPPQPSGVEKAQAM -100
humSULT1C2    - GTTWIQEIVDMIEQNGDVEKCQRAIIQHRHPFIEWARPPQPSGVEKAKAM -100

rabSULT1C2    - PSPRILRTHLPTRLLPPSFWENNCKFLYVARNVKDCMVSYYHFQRMNQVL -150
humSULT1C2    - PSPRILKTHLSTQQLPPSFRENNCEFLYVARNAKDCMVSYYHFQRMNHML -150

rabSULT1C2    - PDPGTWEEYFETFINGKVAWGSWFEHVKGWWEVKGRYQILFLFYEDIKKD -200
humSULT1C2    - PDPGTWEEYFETFINGKVVWGSWFDHVKGWWEMKDRHQILFLFYEDIKRD -200

rabSULT1C2    - PKCEIRKVAQFMGKHLDETVLDKIVQETSFEKMKDNPMINRSTVPKSIMD -250
humSULT1C2    - PKHEIRKVMQFMGKKVDETVLDKIVQETSFEKMKENPMTNRSTVSKSILD -250

rabSULT1C2    - QSISPFMRKGTVGDWKNHFTVAQSHRLDELYRKKMEGVSIDFCLEL     -296
humSULT1C2    - QSISSFMRKGTVGDWKNHFTVAQNERFDEIYRRKMEGTSINFCMEL     -296
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Fig. 2. Alignment of the deduced amino acid sequences from the rabbit and human stomach cDNAs. These sequences share 84.1% identity at the amino acid level. The cytosolic sulfotransferase signature sequences are underlined. The consensus sequences are: TYPKSGTxW (region I) and RKGxxGDWKxxFT (region IV) [3].

Sequence comparison between the rabSULT1C2 and humSULT1C2 clones reveals that the rabbit and human sequences share 83.8% identity at the nucleotide level, which translates to 84.1% identity at the amino acid level. Table 1 shows a comparison of sequence identities (DNA and amino acid) between the newly identified rabSULT1C2 and humSULT1C2 and known cytosolic sulfotransferase sequences. Amino acid identities with two recently reported human sequences hSULT1C1 [25] and human ST1C2 [26] are 84.1 and 100% for rabSULT1C2 and humSULT1C2, respectively. Both the rabbit and human SULT1C2 share around 60% amino acid identity with a previously published rat ST1C1 sequence [28]. Homology with the human hydroxysteroid, estrogen and phenol sulfotransferases is below 60% (Table 1).

Table 1
Nucleotide and amino acid sequence identities of the rabbit (rabSULT1C2) and human (humSULT1C2) sulfotransferases with selected sulfotransferase family members

Sequence	GenBank accession number	Comparative sequence identity (%)			
		rabSULT1C2		humSULT1C2	
		Nucleotide	Amino acid	Nucleotide	Amino acid
rabSULT1C2	AF026304	100	100	83.8	84.1
humSULT1C2	AF026303	83.8	84.1	100	100
hST1C1 [25]	U66036	83.9	84.1	99.9	100
rat ST1C1 [28]	L22339	62.8	60.5	64.7	62.5
humSULT1A1 [13]	L10819	59.2	52.5	60.7	50.2
humSULT1A3 [13]	L19956	64.0	51.9	60.7	49.5
hum liver EST [23]	U08098	57.8	44.9	52.3	44.6
hum DHEA ST [24]	U08024	52.9	31.6	53.7	32.3
PLST [27]	M84135	46.6	22.6	43.1	24.7

Protein expression and immunoblotting

Successful expression of the rabSULT1C2 recombinant protein was achieved using both mammalian COS-cell and bacterial expression systems. The COS-cell expression system was used for the characterisation of the recombinant proteins. Use of the bacterial expression system enabled production of large amounts of recombinant protein for use in production of polyclonal antibodies.

Expressed proteins were separated using SDS-PAGE, transferred onto nitrocellulose membrane and investigated for immuno cross-reactivity using antisera raised against rabSULT1C2. Cross-reactivity between rabSULT1C2 antisera and humSULT1C2 was observed when the recombinant proteins were probed with antisera raised against rabSULT1C2 (Fig. 3). In all cases, the recombinant rabSULT1C2 and humSULT1C2 proteins were observed as a single band corresponding to a molecular weight of 34 kDa. This band was not detected in any of the control lanes (Fig. 3). Very low cross-reactivity was observed when the rabSULT1C2 antisera was used to probe humSULT1A1 and humSULT1A3 recombinant proteins.

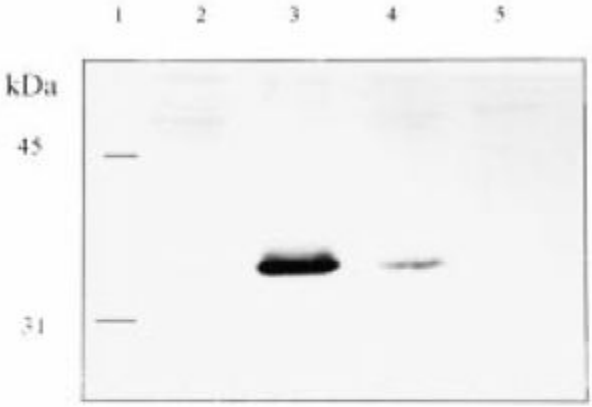

Fig. 3. Immunoblot of expressed recombinant proteins probed with goat anti-rabSULT1C2 (1:200 dilution). Lane 1 shows the migration of Bio-Rad molecular mass markers. Lanes 2 and 5 are controls of BL21 *E. coli* and COS cells transfected with vectors lacking the ST cDNA inserts. Lane 3 shows the bacterial homogenate expressing the rabSULT1C2 (10 μg) and lane 4 the COS-cell homogenate expressing humSULT1C2 (100 μg).

Expression of rabSULT1C2 in tissue cytosols from three individual rabbits was tested by immunoblotting with the rabSULT1C2 antibody. In all tissues examined, rabSULT1C2 migrated as a single band corresponding to a

molecular weight of 34 kDa. Immunoreactivity was observed in rabbit stomach, duodenum, jejunum, ileum, colon, caecum, rectum, kidney and liver (Fig. 4a and b). In contrast, no immunoreactivity was detected in rabbit lung in any of the three rabbit cytosols investigated (results for only one animal shown in Fig. 4b). Expression levels of rabSULT1C2 were high in colon, rectum and kidney, moderate in stomach and duodenum and low in jejunum, ileum, caecum and liver. The levels of rabSULT1C2 expression in the different tissues also differed between the three rabbits investigated.

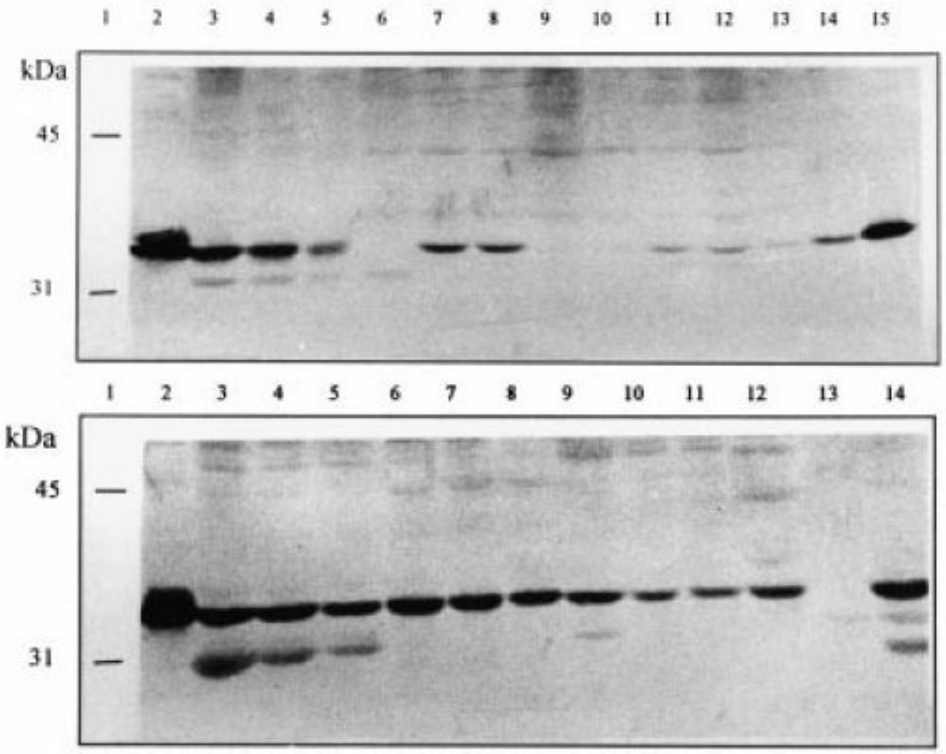

Fig. 4. Immunoblots of rabbit tissue cytosols probed with goat anti-rabSULT1C2 (1:200 dilution). The samples were subjected to SDS-PAGE and probed with the anti-rabSULT1C2 antisera. Lane 1 shows the migration of the Bio-Rad molecular mass markers. (a) Lane 2 and 15 are a homogenate (5 μg protein) of BL21 *E.coli* cells transfected with rabSULT1C2 cDNA and a homogenate (100 μg protein) of COS-7 cell transfected with rabSULT1C2 cDNA, respectively. Rabbit tissue cytosols (150 μg protein) were run in lanes 3–14: lanes 3–5 stomach, lanes 6–8 duodenum, lanes 9–11 jejunum, lanes 12–14 ileum. Cytosols isolated from tissues of three individual rabbits are shown in each tissue grouping. (b) Lane 2 contained a homogenate (5 μg protein) of BL21 *E.coli* cells transfected with rabSULT1C2 cDNA. Rabbit tissue cytosols (150 μg protein) were run in lanes 3–11: lanes 3–5 colon, lanes 6–8 rectum, lanes 9–11 caecum. Cytosols isolated from three individual rabbits are shown in each tissue grouping. Lanes 12–14 contain liver, lung and kidney cytosol (150 μg protein), respectively, from one rabbit.

Enzyme characterisation

Substrate assays

Functional characterisation of the expressed recombinant rabSULT1C2 protein was undertaken using a range of potential substrates that have previously been shown to be sulfonated by other members of the sulfotransferase enzyme superfamily. Of the 16 compounds investigated, sulfonation of only one substrate, p-nitrophenol, by rabSULT1C2 was detected (Table 2). The K_m value for the sulfonation of p-nitrophenol displayed by rabSULT1C2 is 2000-fold greater than that displayed by human SULT1A1, a sulfotransferase that preferentially catalyses this reaction (Table 2). p-Nitrophenol is therefore a relatively poor substrate for rabSULT1C2. Rabbit stomach cytosol and COS-cell expressed rabSULT1C2 displayed similar K_m values for the sulphonation of p-nitrophenol, suggesting that rabSULT1C2 is the predominant form of ST expressed in the rabbit stomach responsible for the sulfonation of p-nitrophenol. The recombinant humSULT1C2 also metabolised p-nitrophenol, and like the rabSULT1C2 enzyme, p-nitrophenol was also a poor substrate for humSULT1C2. The K_m for the sulfonation of p-nitrophenol by

humSULT1C2 was above 10 mM. Compared to the humSULT1A1 which exhibits a K_m of 0.6 μm for the sulfonation of p-nitrophenol both the rabbit and human enzymes exhibit very low affinities for this substrate. Substrate specificities using COS cell expressed proteins are shown in Table 2. These findings were confirmed when bacterially expressed proteins were investigated (results not shown).

Table 2
Kinetic parameters for the sulfonation of *p*-nitrophenol and dopamine by COS-expressed rabbit stomach ST (rabSULT1C2), humSULT1C2 and rabbit cytosols prepared from the stomach and liver. For comparison, data from humSULT1A1 is shown

Enzyme source	*p*-Nitrophenol[a]		Dopamine[a]	
	K_m (μM)	V_{max} (nmol/min/mg)	K_m (μM)	V_{max} (nmol/min/mg)
rabSULT1C2	2200 ± 1175	0.39 ± 0.10	no activity	no activity
Rabbit stomach cytosol	2100 ± 1065	0.19 ± 0.04	no activity	no activity
humSULT1C2	13350 ± 4116	0.005 ± 0.001	no activity	no activity
Rabbit liver cytosol	0.88 ± 0.45	3.1 ± 0.42	600 ± 144	0.28 ± 0.00
humSULT1A1[b]	0.60	7.5	345	0.16

[a] Values represent the computer-derived mean ± S.E. for kinetic parameters obtained from experiments using duplicate determinations.
[b] Data previously published [13].

Thermostability and inhibition by DCNP

Both COS-cell expressed rabSULT1C2 and rabbit stomach cytosol were relatively thermostable, with 50% of their activity towards p-nitrophenol remaining after 15 min at 47 and 45°C, respectively (Fig. 5). This temperature profile is similar to the positive control humSULT1A1 enzyme while humSULT1A3 which is known to be thermolabile lost 50% of its activity at 39°C. rabSULT1C2 and rabbit stomach cytosol were relatively insensitive to DCNP inhibition with IC_{50} values around 1 mM (Fig. 6). This inhibition profile reflected the positive control, humSULT1A3, which is resistant to DCNP inhibition. In contrast, 50% of humSULT1A1 was inhibited at a concentration of around 1 μm. Because of the low activity of humSULT1C2 to p-nitrophenol the thermostability and sensitivity of this enzyme to the inhibitor DCNP was not investigated.

Discussion

In this study we have successfully isolated and partially characterised a rabbit sulfotransferase that is expressed in the GI tract. The new rabbit sulfotransferase was isolated from a rabbit stomach cDNA library. The structural characteristics of the recombinant protein encoded by the isolated cDNA clearly identify it as cytosolic protein belonging to the sulfotransferase supergene family. Cytosolic sulfotransferase enzymes have been shown to contain a number of highly conserved 'signature sequences' within their primary structure [3]. These include an N-terminal sequence, PKSGTxW in region I of the amino acid sequence and a C-terminal sequence, RKGxxGDWKxxFT in region IV, that are hypothesised to be important in binding of the co-factor PAPS [3 and 5]. The amino acid sequence encoded by the isolated rabbit stomach cDNA contain both of these conserved sulfotransferase signature sequences (Fig. 2). In addition to these structural features, the expressed enzyme was capable of sulfonating the model substrate p-nitrophenol.

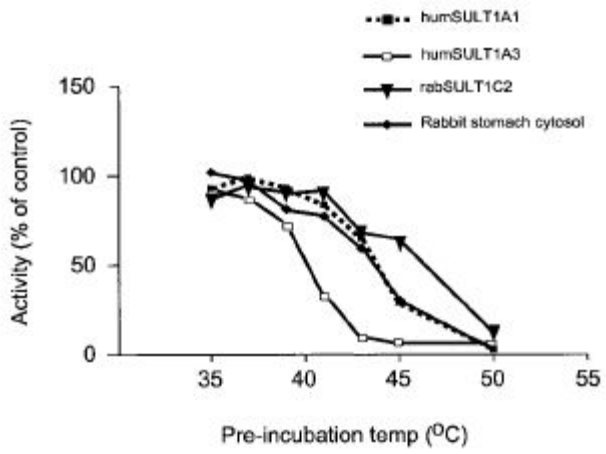

Fig. 5. COS-7 cell homogenates transfected with the humSULT1A1, humSULT1A3, and rabSULTIC2 cDNAs and rabbit stomach cytosol were assayed for activity towards appropriate substrates after pre-incubation for 15 min at given temperature. The COS-7 cell data shown were obtained from a single transfection experiment with each cDNA so as to avoid batch-to-batch variability. The single set of data shown is representative of the findings of five experiments.

Amino acid sequence comparison of the rabSULT1C2 with two recently reported human ST cDNAs [25 and 26] and the human sequence isolated in this study (humSULT1C2), showed sequence identities at the level of 84.1% (Table 1). This high level of identity confirms that the rabbit cDNA we are reporting is most likely the ortholog of the aforementioned human cDNAs. Her et al. [25] called their cDNA hSULT1C1 to indicate it was the human ortholog of a rat ST1C1 cDNA reported by Nagata et al. [28]. However the amino acid identity shared by these two STs is only 62%. Yoshinari et al. [26] named their human cDNA ST1C2 to indicate that it is structurally related to the rat ST1C1 but is not the ortholog. Nucleotide and amino acid sequence comparison of our humSULT1C2 with those reported by Her et al. [25] and Yoshinari et al. [26] show that bases 1–1027 and 4–1027 of our SULT1C2 matched bases 106–1132 of humSULT1C1 and bases 56–1082 of ST1C2, respectively. All three human cDNAs encode the identical sulfotransferase.

Alignment of the rabSULT1C2 and humSULT1C2 amino acid sequences reported in this study with the rat ST1C1 reveals identities of slightly greater than 60% (Table 1). These findings indicate that the rabbit (rabSULT1C2), human (humSULT1C2 [25 and 26]) and rat ST1C1 [28]) sulfotransferases can be grouped within a common sulfotransferase subfamily. For example, amino acid sequence analysis comparing rabSULT1C2 and humSULT1C2 with sequences reported for other cytosolic sulfotransferases reveal sequence identities of around 50% with members of the SULT1 (phenol) family and less than 45% identity with members of the SULT2 family (hydroxysteroid family) (Table 1). Thus, in keeping with the currently accepted nomenclature system recommended for sulfotransferases, rabSULT1C2, humSULT1C2 and rat ST1C1 belong within a distinct sub-family within the SULT1 family of STs. The sequences of two rabbit liver STs: rabbit ST2A8 and ST3A1 [4 and 29] have recently been reported to have very low amino acid sequence identity with members of the phenol ST family. Similarly, these proteins displayed amino acid identity of less than 38% with rabSULT1C2 and humSULT1C2.

Probing of cytosols from a range of rabbit tissues with goat anti-rabSULT1C2 sera recognised a distinct 34 kDa molecular weight protein band in liver, stomach, small intestine, colon, rectum, caecum and kidney samples. These bands co-migrated with the COS cell and E. coli expressed rabSULT1C2. The strongest immunoreactive bands were seen in the colon, rectum and kidney, with moderate bands observed in the stomach and duodenum. Weak immunoreactive bands were detected in the jejunum, ileum, caecum and liver (Fig. 4a and b). These findings clearly demonstrate that rabSULT1C2 is expressed in tissues of the GI tract, with differential levels of expression between the tissue types. The expression of rabSULT1C2 across the GI tract was generally observed to be moderate in the stomach, low in the small intestine and high in the large intestine and rectum. Our findings are in agreement with the mRNA dot blots reported by Her et al. [25], where positive mRNA signals for human SULT1C1 (SULT1C2) sulfotransferase were detected from adult human stomach, kidney and thyroid as well as foetal liver and kidney. Detection of this sulfotransferase in the foetal liver–spleen cDNA libraries by Her et al. [25] and Yoshinari

et al. [26] could indicate age-related expression, as screening of an adult human cDNA liver library by our group did not identify a similar clone (results not shown). An unidentified protein (MW ~31 kDa) also displayed immunoreactivity with anti-rabSULT1C2 antisera. It is possible that this band represents another, as yet unidentified, rabbit sulfotransferase isoform. Alternatively, an unrelated rabbit protein with common epitopes to rabSULT1C2 may be cross-reacting with the polyclonal antibodies.

Characterisation of the substrate specificity of rabSULT1C2 recombinant protein was undertaken and of the 16 substrates tested at both micromolar and millimolar concentrations, only sulfonation of p-nitrophenol was detectable. The expressed rabSULT1C2 protein catalyses the sulfonation of p-nitrophenol with a K_m of 2.2 mM while humSULT1A1 [13] has a K_m for this reaction of 0.6 μM. These data show that p-nitrophenol is a relatively poor substrate for both the rabSULT1C2 and humSULT1C2 enzymes (Table 2). Similar to the findings in this paper for COS expressed humSULT1C2, Yoshinari et al. [26] also observed sulfonation activity of their bacterially expressed human ST1C2 using a concentration of 3 mM for p-nitrophenol. Studies are currently ongoing within our laboratory to identify the physiological or xenobiotic substrate(s) of both the rabbit and human SULT1C2 enzymes.

Previous studies of STs have used inhibitor sensitivity and thermostability to characterise new isoenzymes at the functional level. For example, humSULT1A1 has been shown to be thermostable and sensitive to 2,6-dichloro-4-nitrophenol (DCNP) inhibition while humSULT1A3 has been identified as thermolabile and insensitive to DCNP inhibition [13]. In the present study the rabSULT1C2 was shown to be similar to the humSULT1A1 in its thermostability characteristics (Fig. 5) however, in relation to its sensitivity to DCNP it is more like humSULT1A3 (Fig. 6).

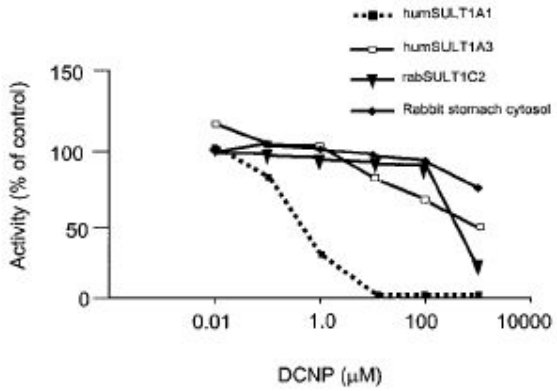

Fig. 6. Inhibition by 2,6-dichloro-4-nitrophenol (DCNP) of COS-7 cell homogenates transfected with the humSULT1A1, humSULT1A3 and rabSULT1C2 cDNAs and rabbit stomach cytosol were measured at various concentration of the inhibitor in the presence of the appropriate substrates. The COS-7 cell data shown were obtained from a single transfection experiment with each cDNA so as to avoid batch-to-batch variability. The single set of data shown is representative of the findings of two experiments.

Studies investigating ST expression in human small intestine have indicated the presence of the thermolabile form of phenol sulfotransferase (humSULT1A3) [30] as well as the estrogen (SULT1E) and dehydroepiandrosterone (DHEA, SULT2) sulfotransferases [31]. Investigation of the specificity of the recombinant rabSULT1C2 protein and rabbit stomach cytosol for the preferred substrates of humSULT1A3 (dopamine), humSULT1E1 (estrone) and humSULT2 (DHEA) revealed that sulfonation activity towards these substrates was undetectable. We have also shown that rabbit stomach cytosol and the rabbit recombinant rabSULT1C2 protein displayed similar K_m values for p-nitrophenol and similar thermostability and DCNP inhibition characteristics. This findings therefore suggest that rabSULT1C2 is the major ST expressed in the rabbit stomach.

This study reports the molecular characterisation of a new rabbit SULT1C2 enzyme and its human ortholog. The latter shows 100% identity with two recently reported human sequences [25 and 26]. The rabSULT1C2 is present in the stomach, duodenum, jejunum, ileum, caecum and rectum of the rabbit. Sequence analysis of rabSULT1C2 and humSULT1C2 show they belong to a common sub-family within the SULT1 (phenol) family. To conform to the current ST nomenclature, we propose the rabbit and human cDNAs reported in this study be termed 1C2 sulfotransferases to indicate they are related to the rat ST1C1 [28] but are not the respective orthologues.

Acknowledgements

This research was supported by the National Health and Medical Research Council of Australia (Grant No. 951291). Substrate studies of rabSULT1C2 with 4-hydroxytamoxifen were carried out by Ms Josephine Maria.

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